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Sequence, conservation, and quantitative expression of rainbow trout *Myf5*

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Abstract

The success of rainbow trout as an aquaculture species is dependent on the ability to produce fish with large amounts of high-quality lean muscle. It is therefore important to understand not only the best conditions under which to raise the fish but also the molecular control of muscle growth. Vertebrate muscle growth is initiated by the specification of myogenic precursor cells into myoblasts. The myoblasts proliferate and fuse to form multinucleated myotubes, which mature into myofibers. A family of basic helix-loop-helix (bHLH) transcription factors, the Myogenic Regulatory Factors (MRFs), controls these events. In trout, two MRF-encoding genes, *TMyoD* (of which there are two) and *Tmyogenin*, have been identified. However, the primary MRF-encoding *Myf5* is not yet sequenced. Here, using degenerate PCR and 5′ and 3′ RACE, the cDNA sequence of trout *Myf5* (*TMyf5*) is identified. Translation of the cDNA reveals that TMyf5 is a bHLH protein with homology to Myf5 and MRFs in other organisms. It is expressed mainly in red and white muscle, suggesting that it shares functional homology to Myf5 in other species. The molecular control of muscle growth has been well-characterized in mammals, but there are differences in the growth of fish muscle, highlighting the need for characterization of MRFs in fish species, particularly those in which understanding muscle growth will have a positive impact on the economic potential of the species.

Keywords: *TMyf* 5; Rainbow trout; MRF; Muscle

1. Introduction

The characterization of muscle development and growth in rainbow trout has become a topic of importance as methods are evaluated to improve the economic potential of the fish. The production of healthy fish strains with large amounts of high-quality lean muscle is of great interest to aquaculturists. To this end, approaches such as varying and supplementing the composition of fish feed (Hardy, 2002), treatment of fish with hormones (Mommsen and Moon, 2001), and even exercising the fish (Johnston and Moon, 1980; Davie et al., 1986) have been tested as means of increasing muscle growth.

Fundamental to the production of high-quality lean muscle in fish is an understanding of muscle development and growth, and its molecular control. In vertebrates, muscle development begins in the embryonic stages as myogenic precursor cells become incorporated within the developing somites. As a response to signaling events, the myogenic precursors become myoblasts, which proliferate and then fuse to form multinucleated myotubes. Myofibers, the functional units of muscle, are formed by the maturation and fusion of myotubes (Currie and Ingham, 1998). In adult myogenesis, which is required for postnatal muscle growth and to repair injured or diseased muscle, satellite cells act as myoblasts for initiation of the myogenic cascade (Seale et al., 2001). A model for vertebrate myogenesis has been delineated in which the Myogenic Regulatory Factors (MRFs), a family of basic helix-loop-helix (bHLH) transcription factors, control key events in the myogenic cascade. The four family members are MyoD, Myf5, Myogenin, and MRF4. MyoD and Myf5 are required for the specification and proliferation of myoblasts (Kablar et

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al., 1999; Rudnicki et al., 1993; Tajbakhsh et al., 1996), Myogenin for the differentiation of myoblasts into multinucleated myotubes (Hasty et al., 1993; Nabeshima et al., 1993), and MRF4 for terminal differentiation that includes myofiber maintenance (Braun and Arnold, 1995; Patapoutian et al., 1995).

The MRF family of transcription factors is very well conserved across species. Despite this conservation, myogenesis in fish and mammals differs in a number of ways. The mammalian myotome, the portion of the somite that gives rise to skeletal muscle, is divided into the epaxial myotome, from which dorsal trunk muscles arise, and the hypaxial myotome, from which limb and abdominal wall muscles arise (Kalthoff, 2001). In fish, since there are no developing limbs (only fin rudiments), there is usually no distinction made between epaxial and hypaxial myotome. The majority of the fish myotome, made up of cells termed lateral presomitic cells, gives rise to white muscle (Currie and Ingham, 1998; Devoto et al., 1996). Adaxial cells are a small group of cells that develop in the somite next to the notochord, migrate through the somite, and give rise to red muscle (Devoto et al., 1996). Another difference is that, in mammals, hyperplasia (proliferation of myoblasts resulting in new myotubes) ceases at birth, and hypertrophy (growth of myotubes and myofibers) accounts for a large percentage of postnatal muscle growth (Parker et al., 2003). In fish, both hyperplasia and hypertrophy continue throughout the lifespan of the fish (Rowlerson and Veggetti, 2001). These differences underscore the importance of MRF characterization in fish as well as in mammals.

Myf5 is considered a primary MRF because it is required for the determination of myoblasts and is also sufficient to drive nonmuscle cells into myogenesis (Tajbakhsh et al., 1996; Braun et al., 1989; Weintraub et al., 1991). In mice, *Myf5* is initially expressed in the epaxial myotome, and in the $Myf5^{-/-}$ knockout, delayed development of epaxial musculature has been observed, suggesting that Myf5 primarily regulates epaxial muscle development (Kablar et al., 1997, 2003). In trout, the genes encoding MyoD and Myogenin have been cloned, but the sequences encoding Myf5 and MRF4 are not yet known. A mutation in *Myf5* does not yet exist in fish, and only preliminary results have been obtained using expression analysis and morpholino injections. In zebrafish, *Myf5* is highly expressed in the lateral presomitic cells and transiently expressed in the adaxial cells (Chen and Tsai, 2002). In morpholino-injected embryos, abnormal muscle development and defective somite patterning have been observed, suggesting that Myf5 plays a similar role in zebrafish and mammals (Chen and Tsai, 2002). Since uncovering the molecular basis of muscle growth is an important step in the improvement of rainbow trout culture, the coding sequence of trout *Myf5* (*TMyf5*) was determined using degenerate PCR, and 5′ and 3′ RACE. In resemblance to its MRF family members, the putative protein sequence contains a bHLH domain, and based on homology with Myf5 in other organisms and on its mRNA expression pattern in trout, most likely functions as a transcription factor in the myogenesis pathway.

2. Materials and methods

2.1. Fish rearing conditions

All fish rearing was carried out at the University of Idaho's Hagerman Fish Culture Experiment Station. Housecreek strain (CSI Hatchery, Idaho) rainbow trout (*Oncorhynchus mykiss*) were raised in 140 L tanks receiving constant temperature 15 °C spring water at a rate of approximately 11.5 L/min, with a 14 h photoperiod until they reached 6 months of age. During the month of July, the fish were moved to a 1317 L outdoor raceway, receiving constant temperature 15 °C spring water at a rate of approximately 400 L/min. Fish were fed a commercial diet 6 days a week, twice daily to apparent satiation. As the fish grew, pellet size was increased according to the ability of the fish to consume the feed. Fish were handled and treated according to the guidelines of the University of Idaho's Animal Care and Use Committee.

2.2. Sample collection and total RNA isolation

Samples from the following tissues of six 11-month old, approximately 500 g rainbow trout were collected: eye, tongue, brain, gills, heart (ventricle), stomach, mid intestine, rear intestine, front kidney, mid kidney, rear kidney, white muscle, red muscle, spleen, adipose tissue, and liver. Two approximately 50 mg samples were collected from each tissue, then total RNA was isolated. All total RNA isolations were carried out using TRIzol according to the manufacturer's protocol (Invitrogen, Rockville, MD).

2.3. Degenerate PCR

The *Myf5* cDNA sequences from zebrafish, sea bass, carp, mouse, and human were aligned, and degenerate primers were designed based on regions of high-sequence conservation. A highly conserved 173 bp region of *TMyf5* was cloned using the degenerate primers. Primer locations and sequences are listed in Fig. 1A and B. RT-PCR was carried out using an MJ PTC-200 and ABgene's Reverse-iT One Step RT-PCR kit (Marsh BioProducts, Surrey, UK). Final concentration of the RT-PCR reaction was 1X RT-PCR Master Mix (contains optimized reaction buffer, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, and 1.25 U Thermoprime Plus DNA Polymerase), 50 U Reverse-iT RTase Blend (includes RNase inhibitor), 100 pmol each Myf5degF2 and Myf5-degR2 (forward and reverse degenerate primers), and 500 ng total RNA isolated from white muscle. Cycling conditions were 30 min at 48 °C, 2 min at 94 °C, 40 cycles

A

B

Primer/probe	nt position	Sequence
1. Myf5degF2	162	TGGGCCTGCAARGCYTGYAAGMG
2. Myf5degR2	334	GCRTTSCKSAGGATCTCYACCTTGGG
3. Myf5GSPFwd	181	AGCGTAAGTCCAGCACAGTGG
4. Myf5nstdGSPFwd	218	CACCATGAGGGAACGACGCC
5. Myf5GSP1	833	GTTGGCAGGTGTTGATCGAT
6. Myf5GSP2	808	CATCACCGAAATAGGAAGTCAAAGC
7. Myf5GSP3	776	CGGTTGAAGATCAGGGCCAT
8. Myf5GSP4	673	TGGGAGTCGGTGCTGGAG
9. Myf5GSP5	641	CATATCTCTGAGCCCTGCTGG
10. Myf5GSP6	619	GCACTGGCATCCACCGA
11. Myf5GSP7	560	GGCACCAGCACCTCTCTCTC
12. Myf5GSP8	464	ACAGTCAACCATGCTGTCGG
13. Myf5GSP9	429	GGCTCCCAGGTTCTGAGCT
14. Myf5AbDFwd	494	CACAAGCTATGGCAACAACTACAG
15. Myf5AbDRev	560	GGCACCAGCACCTCTCT
16. Myf5AbDMGB	542	6FAM-TCCAGAGCTCACATTCT-NFQ
5' AAP	not shown	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP	not shown	GGCCACGCGTCGACTAGTAC
AP	not shown	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
β-actin372F	not shown	TGGCCGTACCACCGGTAT
β-actin451R	not shown	GCAGAGCGTAGTCCTCGTAGATG
β-actin399T	not shown	6FAM-CTCCGGTGACGGCGTGACCC-TAMRA

Fig. 1. Location and sequence of primers and probe used in the cloning and real-time PCR of *TMyf* 5. A representation of the *TMyf* 5 cDNA is shown with the approximate location and direction of primers used in degenerate PCR, 5′ and 3′ RACE, and real-time PCR (A). The cDNA is shown with 5′ to the left, forward primers are represented by right-pointing arrowheads, reverse primers are represented by left-pointing arrowheads, and the probe for real-time PCR is represented as a line. The table lists the nucleotide position and sequence of each primer and probe (B). The name of each primer and probe is listed to the right of the number that corresponds to the labels in panel (A). The nucleotide position corresponds to the 5′-most nucleotide, and each primer and probe sequence is listed 5′ to 3′. The sequences of the degenerate primers (1 and 2) contain the IUB nucleotide ambiguity codes at degenerate nucleotide positions. Primers that are not specific to *TMyf* 5 (β-actin and Invitrogen's RACE primers) are also listed in the table. For the sequence of 5′AAP, "I" refers to deoxyinosine, which has the ability to base-pair with any of the four bases.

of PCR consisting of 20 s at 94 °C, 30 s at 68 °C, and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. PCR products were cloned into Invitrogen's TOPO-TA cloning kit, purified using Qiagen's Spin Miniprep Kit, then sequenced by Fisher's SeqWright service using the dideoxy method.

2.4. 5′ and 3′ RACE

The remaining sequence of *Myf5* was determined using 5′ and 3′ RACE (*R*apid *A*mplification of *c*DNA *E*nds) with reagents from Invitrogen. For 3′ RACE, 1st strand cDNA was synthesized according to the supplied protocol, using 1.0 μg of freshly isolated total RNA from white muscle and 10 pmol of the supplied Adaptor Primer (AP, complementary to the polyA region). Amplification of the synthesized cDNA was carried out according to the supplied protocol, using 10 pmol each of a *Myf5* gene specific primer (Myf5GSPFwd) and the supplied AUAP (primer specific for the sequence added by the AP). Cycling conditions were 3 min at 94 °C, 32 cycles of PCR consisting of 30 s at 94 °C, 30 s at 59 °C, and 1.5 min at 72 °C, then a final elongation step of 10 min at 72 °C. An approximately 900 bp fragment was obtained, then 2 μl of this amplification product was used as template in a nested amplification reaction. Reaction and cycling conditions were the same as in amplification of 1st strand cDNA, except that the primers used were Myf5nestedGSPFwd and AUAP (10 pmol each), and the annealing temperature during PCR was 66 °C. A fragment of approximately 850 bp was obtained, cloned using Invitrogen's TOPO-TA cloning kit, purified using Qiagen's Spin Miniprep Kit, then sequenced by Fisher's SeqWright service using the dideoxy method. Primer locations (for *TMyf5* primers) and sequences are listed in Fig. 1A and B.

5′ RACE was carried out according to the protocol included in Invitrogen's 5′RACE kit, with modifications noted below. 1st strand cDNA was generated using 450 ng polyA+ mRNA and 10 pmol each of four *Myf5* gene-specific primers (Myf5GSP1, Myf5GSP2, Myf5GSP3,

Myf5GSP4). After synthesis, the reactions were purified using Qiagen's PCR Purification kit, then tailed with 5 pmol dCTP, using 45 U of Promega TdT. Amplification of dCTP-tailed cDNA was carried out using 20 pmol of a *Myf5* gene-specific primer (Myf5GSP5) and 30 pmol of the supplied 5′Abridged Anchor Primer (5′AAP, complementary to dCTP-tailed region). Cycling conditions were 2 cycles PCR consisting of 5 min at 94 °C, 2 min at 56 °C, and 2 min at 72 °C, then 33 cycles of PCR consisting of 30 s at 94 °C, 30 s at 56 °C, and 1.5 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. An approximately 620 bp band was detected in all amplification samples, and each selected fragment was gel-purified using Qiagen's gel extraction kit (Qiagen, Valencia, CA). The gel-purified fragments were pooled and used as template in subsequent nested PCR amplifications. Reaction and cycling conditions were the same as in the amplification of the dCTP-tailed cDNA, except that the primers used were *Myf5* gene-specific primers (Myf5GSP6, Myf5GSP7, Myf5GSP8, Myf5GSP9) and the supplied AUAP (primer specific for the sequence added by the 5'AAP; 20 pmol each). Amplification products were obtained, whose size corresponded to the location of each *Myf5* gene-specific primer. The product obtained using MYF5GSP9+AUAP (approximately 430 bp) was cloned using Invitrogen's TOPO-TA cloning kit, purified using Qiagen's Spin Miniprep Kit, then sequenced by Fisher's SeqWright service using the dideoxy method. Primer locations (for *TMyf5* primers) and sequences are listed in Fig. 1B.

2.5. Real-time quantitative RT-PCR

To detect the level of *TMyf5* and *β-actin* gene expression in each tissue (eye, tongue, brain, gills, ventricle, stomach, mid intestine, rear intestine, front kidney, mid kidney, rear kidney, white muscle, red muscle, spleen, adipose tissue, and liver), real-time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI (Foster City, CA), according to the protocol provided by ABI. The final concentration of each reaction was Master Mix, 1× (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/μL; RNase inhibitor mix, 0.4 U/μL; forward primer, 900 nM; reverse primer, 900 nM; probe, 250 nM; total RNA, 50 ng. *TMyf5* primers and probe were designed by ABI's Assay by Design service. Cycling conditions for *TMyf5* were as follows: 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of PCR consisting of 15 s at 95 °C followed by 1 min at 60 °C. As a cellular mRNA control, *β-actin* levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999). *β-actin* primers and probe were designed using Primer Express software (ABI). Cycling conditions for *β-actin* were 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, then 40 cycles of PCR consisting of 20 s at 95 °C followed by 1 min at 62 °C. All samples were run in duplicate. Primer and probe sequences for real-time PCR are listed in Fig. 1B. For *TMyf5*, real-time primers and probes are numbers 14, 15, and 16, and for β-actin, real-time primers and probes are called β-actin372F, β-actin451R, and β-actin399T, respectively.

Absolute copy number of each mRNA sample tested was determined by including standards consisting of in vitro transcribed mRNAs specific for *TMyf5* or *β-actin*, with each set of experimental samples that were analyzed by real-time quantitative RT-PCR. To make standards, the same primers designed for real-time amplification were used in RT-PCR of total RNA isolated from white muscle tissue to amplify a fragment of *TMyf5* or *β-actin*. The fragments were cloned using Invitrogen's TOPO-TA cloning kit, then sequenced to determine orientation of insertion. Standards were generated by in vitro transcription of the *TMyf5* or *β-actin* clone using Promega's Riboprobe in vitro Transcription System (Madison, WI). The transcripts were run on formaldehyde/MOPS gels to confirm the presence of a single band of the correct size, then quantified using a spectrophotometer. The molecular weight of the in vitro transcribed RNA was calculated using the following formula: MW=(# of A bases×328.2)+(# of U bases×305.2)+(# of C bases×304.2)+(# of G bases×344.2)+159. Using the MW and concentration, copy #/μL was determined. These transcripts were used as quantitative standards to determine absolute mRNA copy number in each experimental sample. Five serial dilutions of standards were used for each run to generate the standard curve. All quantities were based off of an initial quantification of the isolated in vitro transcribed RNA. As such, the absolute numbers given correspond accurately between each run.

2.6. Data analysis

The real-time expression data are reported as a ratio of absolute mRNA copy number of *TMyf5* to the absolute mRNA copy number of *β-actin*, multiplied by a constant variable for ease of interpretation, and expressed as means ± standard errors. Microsoft Excel was used to produce graphical representations of the data. Statistical analysis of the data was performed using Statistica6 (StatSoft). One-way ANOVA was used with Newman–Keuls for pairwise comparisons between tissue and *TMyf5* gene expression. Significance levels were set at $P<0.05$.

3. Results

3.1. Nucleotide and predicted protein sequence of TMyf5

Nucleotide sequences of the zebrafish, carp, striped sea bass, mouse, and human *Myf5* cDNA were aligned, and degenerate primers were designed in regions of high homology. A 173 bp region of *TMyf5* was amplified from

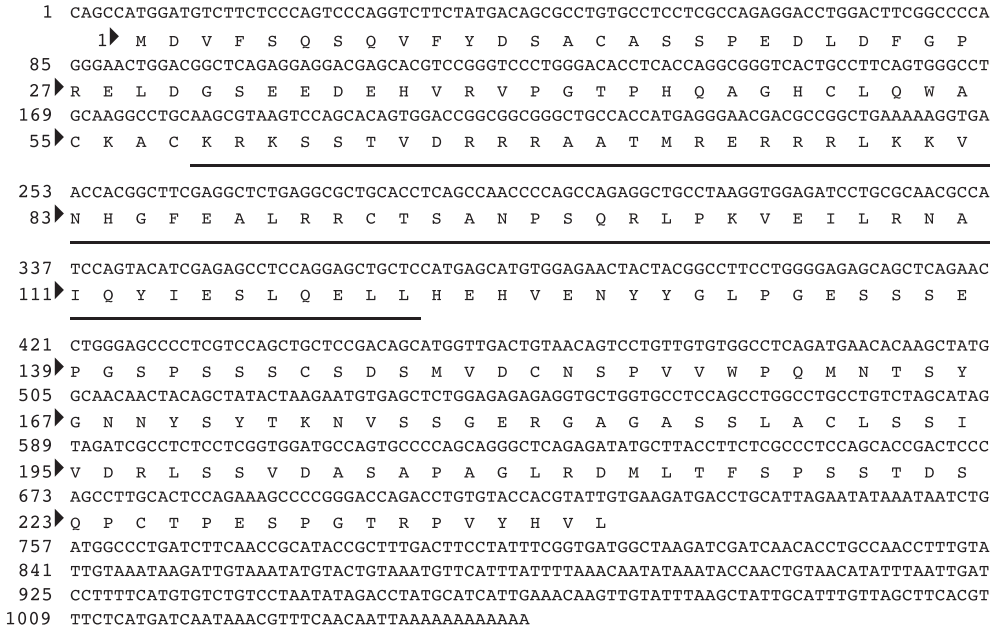

Fig. 2. Nucleotide and amino acid sequence of TMyf5. The nucleotide sequence of *Myf5*, beginning 5 bases upstream of the coding sequence, is listed 5′ to 3′. The sequence has been deposited in Genbank with the accession number AY751283. The ExPASy translation tool (Swiss Institute of Bioinformatics) was used to generate a putative amino acid sequence of Myf5. The protein is predicted to be a 239 amino acid polypeptide that contains a bHLH domain (underlined).

white muscle total RNA, using the degenerate primers Myf5degF2 and Myf5degR2 (Fig. 1A, B). Rapid Amplification of cDNA Ends (RACE) was used to determine the remaining sequence of *TMyf5*. 3′ RACE yielded an approximately 850 bp fragment, containing partial coding sequence and the 3′UTR. 5′RACE yielded a product of approximately 434 bp, which consisted of the remaining unknown coding sequence plus 5 bp of 5′ UTR. Combining the sequence obtained by degenerate PCR, 3′ RACE, and 5′RACE, a 1050 bp region of *TMyf5* cDNA sequence was determined, which contained the 720 bp coding region (Fig. 2). This sequence has been deposited in Genbank with the accession number AY751283.

The *TMyf5* coding sequence was translated to a putative protein sequence using ExPASy (Expert Protein Analysis System, Swiss Institute of Bioinformatics). The TMyf5 protein is predicted to be a 239 amino acid polypeptide (Fig. 2) containing a basic helix-loop-helix (bHLH) domain

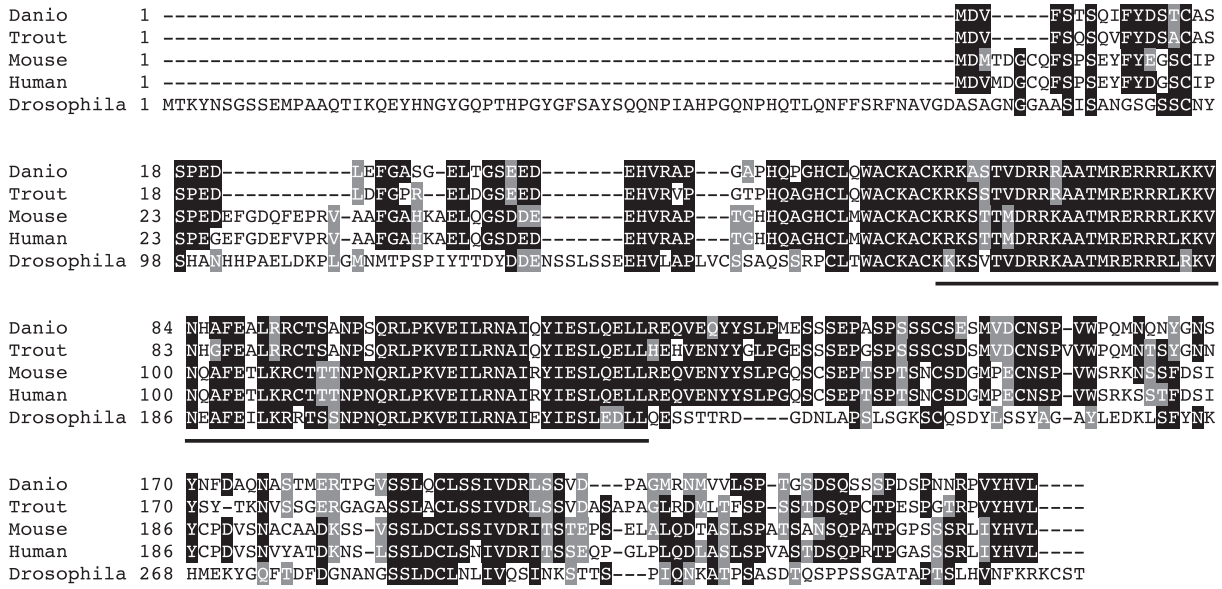

Fig. 3. Alignment of the TMyf5 amino acid sequence with Myf5 from other species. The putative amino acid sequence of trout Myf5 is compared to the amino acid sequences of zebrafish, mouse, and human Myf5, and *Drosophila* Nautilus. The alignment was generated using CLUSTAL and Boxshade. Identical and similar residues are shaded black and gray, respectively. The aligned bHLH domains are indicated by an underline.

Fig. 4. Alignment of the rainbow trout MRF proteins. The putative amino acid sequence of trout Myf5 is aligned with the other known trout MRF-family proteins MyoD1, MyoD2, and Myogenin. The alignment was generated using CLUSTAL and Boxshade. Identical and similar residues are shaded black and gray, respectively. The aligned bHLH domains are indicated by an underline.

(Fig. 2, underlined portion). bHLH domains are present in some transcriptional regulators and mediate sequence specific DNA binding as well as dimerization with other proteins (Puri and Sartorelli, 2000), indicating that TMyf5, like its homologs in other species, is likely a transcriptional regulator that functions in a multiprotein complex.

3.2. Conservation of TMyf5

Previously characterized Myf5 proteins from several organisms have been shown to share sequence identity with each other and have similar function (Rescan, 2001). An alignment of the amino acid sequence of several Myf5 homologs reveals that the entire TMyf5 protein shares approximately 80%, 61%, 58%, and 41% identity with zebrafish, mouse, human, and *Drosophila* Myf5, respectively (Fig. 3; in *Drosophila*, there is one muscle-specific bHLH protein, Nautilus). The bHLH domain, which confers function to the protein, is 96%, 82%, 82%, and 77% identical between TMyf5 and zebrafish, mouse, and human Myf5, and *Drosophila* Nautilus, respectively (Fig. 3), suggesting that the function of TMyf5 is similar to the known function of Myf5 in other organisms, i.e., a transcriptional regulator that controls myoblast specification and proliferation.

3.3. Conservation of the trout MRF proteins

The highly conserved vertebrate MRF family consists of MyoD, Myf5, Myogenin, and MRF4, and in those species

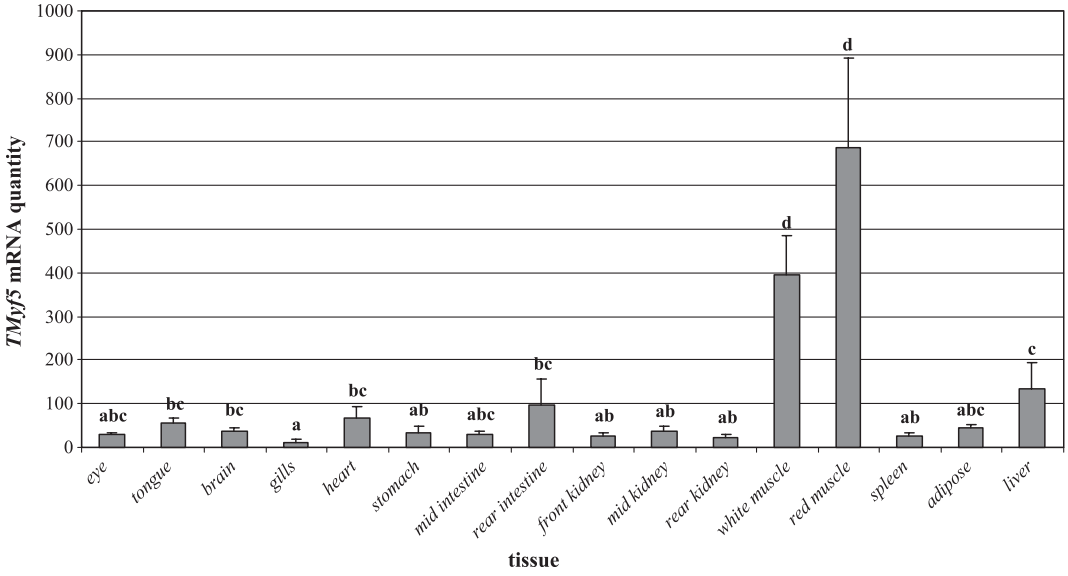

Fig. 5. Expression of TMyf5 in selected tissues. Mean±S.E. of *TMyf*5 mRNA quantity is shown for each tissue tested. Quantities were determined by dividing the absolute copy number of *TMyf*5 mRNA by the absolute copy number of *β-actin* mRNA in each sample, then multiplying by a common factor for ease of interpretation. Quantities that are significantly different at $P<0.05$ are labeled with different letters.

for which the myogenic pathway has been characterized, each plays a major role. In trout, the sequences of *MyoD* (of which there are two, *TMyoD1* and *TMyoD2*), *Tmyogenin*, and now *TMyf5*, are known, and as is the case in species in which the sequences of MRF-encoding genes are known, the bHLH domains of trout MRFs are conserved. An alignment of TMyf5 with the other known trout MRF proteins reveals that there is 76%, 77%, and 74% identity between the TMyf5 and the TMyoD1, TMyoD2, and TMyogenin bHLH domains, respectively (Fig. 4).

3.4. Expression of TMyf5

The embryonic expression pattern of *Myf5* in zebrafish has been characterized, as has the expression pattern of *Myf5* in embryonic and adult mouse tissue. However, information on *Myf5* expression in adult fish tissues is lacking. In an effort to better analyze *TMyf5*, quantitative real-time RT-PCR was used to determine the expression levels of *TMyf5* in a number of tissues from approximately 500 g adults. *TMyf5* shows the highest level of expression in red muscle, with expression in white muscle appearing slightly lower (Fig. 5), but the difference in these levels is not statistically significant. Liver and rear intestine are the next highest-expressing tissues, showing approximately 18%–25% of the expression found in red and white muscle (Fig. 5). The expression level of *TMyf5* in the remaining tissues was extremely low compared to muscle, ranging from approximately 3% in the gills to 13% in the ventricle. These expression patterns suggest that TMyf5 plays a major role in muscle development and maintenance, and little or no role in the other tissues for which expression was tested.

4. Discussion

The conservation of *TMyf5* sequence and its expression pattern in muscle indicate that its function may be similar to that found in other studied systems. TMyf5 contains a bHLH domain that is 82% identical to the bHLH domains of both mouse and human Myf5, in which the function of Myf5 has been well-established as a transcription factor that initiates cellular entry into the myogenic pathway. In several organisms, including zebrafish and carp, *Myf5* is the first of the MRF-encoding genes to be expressed during embryogenesis, initiating expression of the remaining MRFs (Kobiyama et al., 1998; Rescan, 2001). Transfections in human cell lines have suggested that Myf5 exerts its function by remodeling the chromatin at binding sites in the enhancers of muscle-specific genes such as *myogenin* and *muscle creatine kinase*, allowing transcriptional activation of the genes (Gerber et al., 1997; Bergstrom and Tapscott, 2001). The domains that confer the chromatin remodeling activity to human Myf5 are also conserved in TMyf5, suggesting that it too may function as a chromatin modifier in the enhancers of muscle-specific genes.

The majority of information on *Myf5* expression in fish has been collected from the embryonic stages. In zebrafish, *Myf5* is expressed in the segmental plates (from which somites arise) and in the lateral presomitic cells, the part of the somite from which white muscle arises (Chen et al., 2001). It is also expressed transiently in the adaxial cells, from which red muscle arises (Chen et al., 2001). In adult trout, *TMyf5* shows strong expression in both red and white muscle, while expression in other tissues is much lower. If trout embryonic expression is similar to that of zebrafish, then it is likely that *TMyf5* is expressed in the lateral presomitic cells and transiently in the adaxial cells. Expression must then be resumed in the red muscle after the adaxial cells have migrated through the somite to form the red muscle. In situ hybridization to embryos would confirm this hypothesis.

In mammals, *Myf5* expression and function is initially limited to the epaxial myotome, which gives rise to the dorsal trunk muscles, while *MyoD* expression and function is initially limited to the hypaxial myotome, which gives rise to the limb muscles (Kablar et al., 1997, 2003). Since hypaxial myotome is greatly diminished in fish, it is tempting to hypothesize that Myf5 plays the major role in the specification of myoblasts, while the role of MyoD is minor, only functioning in the reduced hypaxial myotome. However, this does not seem to be the case. It is true that *MyoD* expression has been shown in the zebrafish fin bud, originating from ventral (hypaxial) somitic cells (Neyt et al., 2000). However, in the fish species for which it has been tested, *MyoD* expression is not restricted to fin buds. In trout, there are two *MyoD* genes; *TMyoD1* is expressed in the adaxial cells, then expands laterally through the somites, and *TMyoD2* is expressed in all cells of the posterior domain of each newly formed somite (Delalande and Rescan, 1999). Also, *TMyoD1* and *TMyoD2* expression in trout has been found in both red and white muscle throughout development (Delalande and Rescan, 1999; Johansen and Overturf, submitted for publication). While these expression patterns do not definitively prove that MyoD is required for fish myogenesis, it seems unlikely that Myf5 alone plays the major role in myoblast specification.

Single and double *Myf5* and *MyoD* loss-of-function mutants need to be generated and analyzed in fish to determine the possibility that Myf5 and MyoD have functionally redundant but spatially distinct roles in fish myogenesis, as has been shown in mammals. So far, the only *Myf5* loss-of-function analysis in fish has been the treatment of zebrafish embryos with *Myf5* morpholino (Chen and Tsai, 2002). This resulted in decreased *myogenin* expression, supporting the thought that *myogenin* is a direct transcriptional target of Myf5. *Myf5* morpholino-treated embryos also showed abnormal epiboly, abnormal tail fins, U-shaped somites, and small heads (Chen and Tsai, 2002). These phenotypes are more severe than those noted in

mammals, prompting the authors to suggest that Myf5 performs more and different functions in zebrafish than it does in mammals (Chen and Tsai, 2002). However, it is difficult to know whether these "knock-down" phenotypes are a true representation of the phenotypes that would arise as a result of a stably integrated "knock-out" mutation. Another variable in determining the function of *TMyf5* is the possibility that there is more than one *Myf5* gene in trout, which may have arisen as a result of a genome duplication event that is thought to have occurred in some fishes (Rescan, 2001). Indeed, within the group of genes thought to control muscle development in rainbow trout, there are two *MyoD* genes (Rescan and Gauvry, 1996), and two *myostatins* (Rescan et al., 2001).

In those species in which both *Myf5* and *MRF4* have been mapped, it has been found that they are linked (Braun et al., 1990; Saitoh et al., 1993; Patapoutian et al., 1993; Cupelli et al., 1996). In fact, the genomic sequences of *Myf5* and *MRF4* overlap in mice (Carvajal et al., 2001). If this situation is true in rainbow trout, then it should be relatively easy to identify the sequence of *MRF4*. Based on distances between the two in other vertebrates, it is likely that *TMyf5* and *TMRF4* are located very close to each other, if not overlapping. The enhancer region of mouse *Myf5* has been well studied, revealing the control of *Myf5* domain-specific expression (Summerbell et al., 2000; Hadchouel et al., 2000, 2003; Carvajal et al., 2001). The proximity of *Myf5* and *MRF4*, and somewhat overlapping expression patterns, suggest that they may share enhancer elements. The 140 kb region upstream of *Myf5* contains all enhancer elements responsible for both *Myf5* and *MRF4* expression, and it is likely that the aspects of expression that each gene shares are controlled by sequences in the same genomic region (Carvajal et al., 2001). A partially shared enhancer region may be the basis for the tight linkage of the genes seen across species (Carvajal et al., 2001). If the enhancer regions of *TMyf5* and *TMRF4* are shared, it is likely that the expression of *TMRF4* will be at least partially similar to that of *TMyf5*.

Continued expansion in aquaculture will rely on the development of fish stocks with improved health and protein content characteristics. Flesh quality is determined by a number of factors such as texture, color, fat content, and also by handling procedures during processing (Johnston, 1999). Among these, texture is dependent on muscle cellularity, and a basic understanding of the molecular processes that lead to muscle development and maintenance can help in achieving improved texture, and in turn, improved flesh quality (Johnston, 1999). A great deal is already known about the molecular control of muscle development in mammals, but information on muscle development in fish is not as abundant. The emergence of zebrafish as a genetic model of development has provided some clues into the differences between mammalian and fish muscle development. However, zebrafish do not grow markedly after the juvenile stages, and it is therefore vital to investigate the molecular control of muscle development in commercially valuable species, such as rainbow trout, that do show marked postjuvenile growth. The cloning and characterization of the MRFs is a step toward molecular understanding, and the information provided here should be an important addition.

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